PB86-188208

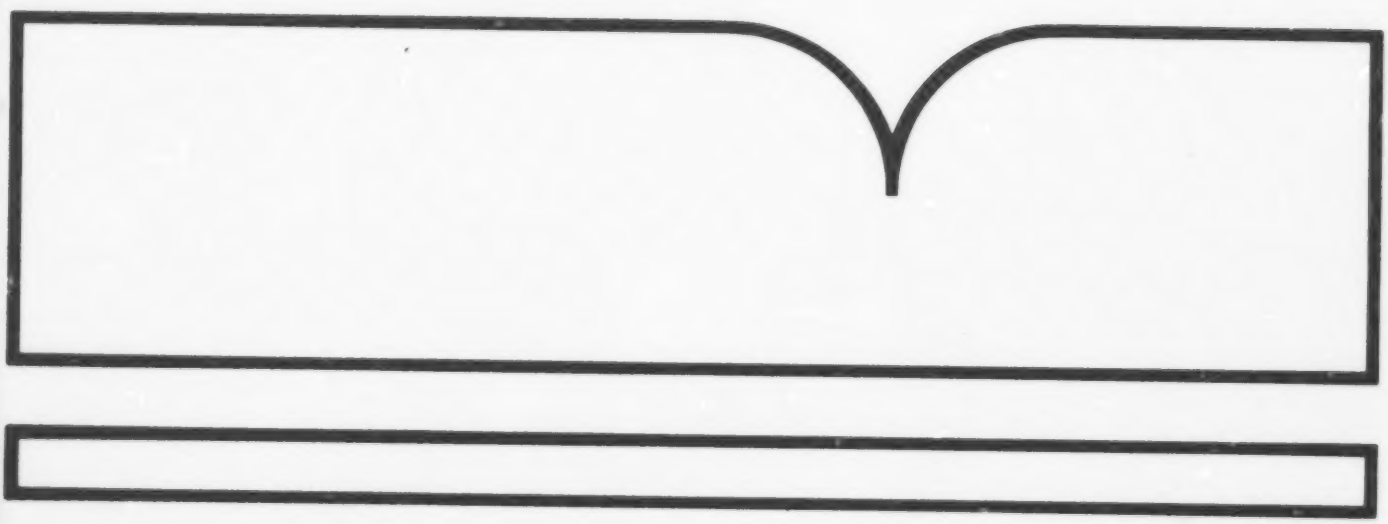
Basis of the Insensitivity of 'Salmonella typhimurium' Strain TA98/1,8-DNP6 to the Mutagenic Action of Nitroarenes

Case Western Reserve Univ., Cleveland, OH

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1983



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1. REPORT NO. EPA/600/J-83/336	2.
3. R PB86-188208	
4. TITLE AND SUBTITLE The basis of the insensitivity of Salmonella typhimurium strain TA98/1,8-DNP6 to the mutagenic action of nitroarenes (Journal Version)	5. REPORT DATE 1983
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) E.C. McCoy, M. Anders and H.S. Rosenkranz	8. PERFORMING ORGANIZATION REPORT NO CNNA1E
9. PERFORMING ORGANIZATION NAME AND ADDRESS Case Western Reserve Univ., Cleveland, OH	10. PROGRAM ELEMENT NO
	11. CONTRACT/GRANT NO CR 809231
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES Mutation Research, 121(1):17-23, July 1983	

16. ABSTRACT

Salmonella typhimurium tester strain TA98/1,8-DNP$_6$ is resistant to the mutagenicity of 1,8-dinitropyrene because it lacks an esterification enzyme which is needed for the formation of the ultimate mutagen, presumably the corresponding hydroxamic acid ester. This enzyme does not appear to be required for the activation of all nitroarenes and arylamines, as some of these are fully active in TA98/1,8-DNP. It is suggested that these form electrophilic arylnitrenium ions non-enzymatically from nitroso- and N-hydroxylamino-arenes intermediates. The esterification enzyme appears to be a transacetylase. An assay using 2-aminofluorene as the acetyl acceptor is described. Derivatives of S. typhimurium TA100 also lacking this enzyme were obtained by Tn5-mediated mutagenesis.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field Group

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18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO OF PAGES 8
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA/600/J-83/336
JOURNAL ARTICLE

Mutation Research, 121 (1983) 17–23
Elsevier

MRLett 0402

The basis of the insensitivity of *Salmonella typhimurium* strain TA98/1,8-DNP$_6$ to the mutagenic action of nitroarenes

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(Accepted 9 March 1983)

Summary

Salmonella typhimurium tester strain TA98/1,8-DNP$_6$ is resistant to the mutagenicity of 1,8-dinitropyrene because it lacks an esterification enzyme which is needed for the formation of the ultimate mutagen, presumably the corresponding hydroxamic acid ester. This enzyme does not appear to be required for the activation of all nitroarenes and arylamines, as some of these are fully active in TA98/1,8-DNP. It is suggested that these form electrophilic arylnitrenium ions non-enzymatically from nitroso- and *N*-hydroxylamino-arenes intermediates. The esterification enzyme appears to be a transacetylase. An assay using 2-aminofluorene as the acetyl acceptor is described. Derivatives of *S. typhimurium* TA100 also lacking this enzyme were obtained by Tn5-mediated mutagenesis.

Strain TA98/1,8-DNP$_6$, now widely used for the detection of nitroarenes, was isolated (McCoy et al., 1981b) as resistant to the mutagenicity of 1,8-dinitropyrene (1,8-DNP). The rationale for the isolation of this strain derived from the finding that while 1,8-DNP was the most potent direct-acting mutagen for Salmonella, it retained its full mutagenicity for TA98NR, a strain deficient in the classical nitroreductase which is required for the expression of the mutagenicity of nitrofurans, niridazoles, nitrofluorenes, nitronaphthalenes and 1-nitropyrene (Mermelstein and Rosenkranz, 1983; Mermelstein et al., 1981; Rosenkranz et al., 1980). It was hypothesized, at first, that 1,8-DNP was capable of reacting directly

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with the cellular DNA without a requirement for the reduction of the nitro function (Mermelstein et al., 1981). However, extensive studies with isolated DNA excluded the possibility of a reaction between 1,8-DNP and DNA (either through intercalation between DNA base pairs or the formation of covalent adducts). This suggested that nitroreduction was required but that it was dependent upon an enzyme other than the classical nitroreductase. This led to the construction of TA98/1,8-DNP_6. The strain was isolated as resistant to the mutagenicity of 1,8-DNP and showed cross-resistances to the mutagenicity of 1,3-DNP and 2-nitrofluorene (McCoy et al., 1981b). It was, however, sensitive to the mutagenicity of 4-nitroquinoline-1-oxide (NQO), 1,3,6-trinitropyrene, 1,3,6,8-tetranitropyrene, niridazole and the

TABLE 1

MUTAGENICITY OF NITROARENES AND RELATED CHEMICALS

Chemical	Activity expressed as mutants per microgram					
	TA98	TA98NR	TA98/1,8-DNP_6	TA100	TA100-Tn5-1,8-DNP 1011	TA100-Tn5-1,8-DNP 1012
2-Nitrofluorene	82.7	0.4	0.2	265	0.84	0.77
2-Nitrofluorene	1592	1406	263	77.6	33.0	11.8
N-Hydroxy-2-aminofluorene	2974	3203	261	138.4	40.6	25.2
N-Acetoxy-2-acetylamino-fluorene	37	31	32	3.5	2.5	6.4
4-Nitroquinoline-1-oxide	820	649	690	4357		5704
Entozon	699	615	535	18.9		20.3
4-Nitro-*o*-phenylenediamine	25.0	13.4	0.6	9.6	0	1.4
1-Nitropyrene	1835	132	530	289		88
1,8-Dinitropyrene	272000	280000	2188	16710	100	716
1,3,6,8-Tetranitropyrene	9761	9907	9605	343	387	335
5-Nitroacenaphthene	23	14	3	9.4	6.2	6.5
2-Aminoanthracene + S9	2559	2830	129	1850	586	460
2-Aminofluorene + S9	852	700	84	319	82	64
2,4-Diaminoanisole sulfate + S9	22.5	24.4	0.2			
Sodium azide				49.7	57.6	55.8
Quercetin	3.0	3.6	3.3	12.2		12.2
Niridazole	1654	14	1714	2151		2044

The mutagenicity was determined as described by Ames et al. (1975). Every chemical was tested on at least two separate occasions. Every dose level was determined in triplicate. Mutagenic activity was calculated from the linear (ascending) portion of the dose–response curve. S9 was derived from the livers of Syrian hamsters (Jackson Laboratories). The origin of most chemicals has been described previously (McCoy et al., 1981a, 1982; 1983; Mermelstein et al., 1981). 2,4-Diaminoanisole sulfate was obtained from the National Cancer Institute Chemical Repository at ITT Research Institute, Chicago (IL).

nitroacridine Entozon (Rosenkranz et al., 1981; McCoy et al., 1981a, b; Mermelstein et al., 1982, see also Table 1). Subsequent studies established that the genetic lesion in TA98/1,8-DNP$_6$ was different from that of TA98NR and that, therefore, it did not involve the classical nitroreductase (Rosenkranz et al., 1982).

It could be deduced, however, that since both TA98NR and TA98/1,8-DNP$_6$ are still sensitive to the mutagenicity of certain nitropyrenes, NQO and Entozon, and as these chemicals require nitroreduction for the expression of their mutagenicity, that Salmonella possesses additional enzymes that participate in the activation of nitro-containing chemicals.

Strain TA98/1,8-DNP$_6$ has proven useful in the detection and subsequent identification of nitroarenes in environmental samples. It has been of special value in the identification of 1,8-DNP in diesel emissions (see Rosenkranz, 1982). It was assumed that TA98/1,8-DNP$_6$ was deficient in a nitroreductase which was different from the classical enzyme.

Recent studies, however, led us to suspect that in actuality the block in TA98/1,8-DNP$_6$ did not involve nitroreduction but a subsequent step in the activation pathway. Thus, unlike the strains deficient in the classical nitroreductase (e.g., TA100NR and TA98NR) mutagenicity was not restored when nitroso- and *N*-hydroxylamino-arenes were used as test compounds. The hydroxamic acid ester, on the other hand, was active in TA98, TA98NR and TA98/1,8-DNP$_6$ (McCoy et al., 1982, see also Table 1). This led to the conclusion that the block in TA98/1,8-DNP$_6$ involved esterification of arylhydroxylamines to hydroxamic acid esters. These findings, which were derived primarily from experiments with 2-amino-, 2-hydroxylamino-, 2-nitroso- and 2-nitro-fluorene, suggested that in addition to the

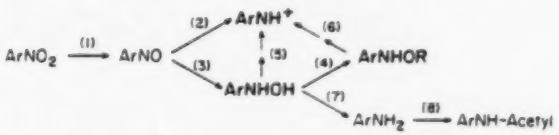

Fig. 1. Biotransformation of nitroarenes in bacteria. (The bold-lettered intermediates are believed to be ultimate mutagens and/or DNA-reactants.) Reaction 1, the nitroreduction can be catalyzed by several nitroreductases including (a) the classical enzyme, (b) the enzyme which catalyzes the nitroreduction of NQO, and (c) the enzyme that acts on 1,8-DNP. Reaction 3, is catalyzed by a nitroso-reductase, an enzyme that is different from the classical nitroreductase as evidenced by the full activity of nitrosoarenes in strains TA100NR and TA98NR. Depending on the nature of the aromatic moiety, nitrosoarene or *N*-hydroxylaminoarenes can be converted non-enzymatically to reactive nitrenium ions (reactions 2 and 3). In addition, the hydroxylamine may require esterification to a hydroxamic acid ester (reaction 4) to express its mutagenicity and this may be accomplished by the esterification enzyme (possibly the transacetylase described herein (e.g., 1,8-DNP). This electrophilic ester may then dismutate non-enzymatically (reaction 6) to yield a reactive nitrenium ion. In addition, the arylhydroxylamine may be further reduced enzymatically to the nonmutagenic arylamine (reaction 7). The further acetylation (reaction 8) may be catalyzed by the same enzyme (a transacetylase) which esterifies the hydroxylamine (i.e., reaction 4). (This reaction is described in the text).

presence of an esterification enzyme, which appeared to be essential for maximal expression of the mutagenicity of some nitroarenes such as 2-nitrofluorene, 4-nitro-*o*-phenylenediamine, 5-nitroacenaphthene and 1,8-DNP, there were additional pathways involved in the expression of the mutagenicity of nitroarenes and arylamines (McCoy et al., 1982, 1983). The results also indicate the existence of a nitroreductase which acts on 1,8-DNP as obviously in order to be esterified the nitro function of 1,8-DNP has to be reduced and this is not accomplished by the classical nitroreductase (as evidenced by the fact that 1,8-DNP expresses its full mutagenicity in TA98NR).

If it be assumed further that the putative esterification enzyme that is deficient in TA98/1,8-DNP$_6$ is capable of acting on a variety of arylhydroxylamines, then the

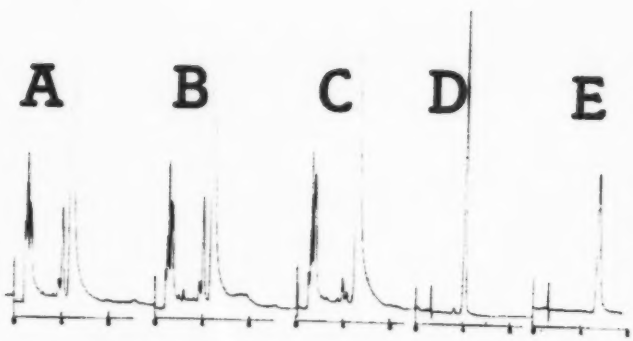

Fig. 2. HPLC profiles of extracts from bacterial cells exposed to 2-aminofluorene: (A) TA98; (B) TA98NR; and (C) TA98/1,8-DNP$_6$. Panels D and E represent the elution profile of 2-acetylaminofluorene and 2-aminofluorene, respectively. Note the great diminution in the peak representing 2-acetylaminofluorene (retention time: 4.0 min) in the extract derived from TA98/1,8-DNP$_6$ (C). Portions (2.5 ml) of bacterial suspensions (approximately 1.6×1.0^8 per ml of 0.067 M phosphate buffer, pH 7.4, containing 1.6% glucose) were incubated for 90 min at 37°C with 2-aminofluorene (final concentration: 25 μg/ml) whereupon the cells were harvested, washed and resuspended in 1 ml of phosphate buffer. They were disrupted by sonication, an equal volume of cold acetone was added and the mixture extracted twice with 2 vol. of methylene chloride. Dehydration was achieved by the addition of 1 g of sodium sulfate. The supernatant fluids were brought to dryness and resuspended in 100 μl of methanol. These preparations were analyzed with a Varian 5000 HPLC apparatus with an ultraviolet detector. The column used was a C_{18} Microbondapak MCH_{10} (0.4 × 30 cm) and isocratic elution was performed with 60% acetonitrile/water. The flow rate was 2 ml/min at 25°C.

the findings suggest that while some nitroarenes such as 1,8-DNP, 2-nitrofluorene are almost entirely dependent upon esterification to hydroxamic acid esters for expression of their mutagenicity, others such as 1,3,6,8-tetranitropyrene (which exerts almost full activity in TA98/1,8-DNP$_6$) form hydroxylamines which either are themselves potent electrophiles or become so following non-enzymatic conversion to the arylnitrenium ion (Fig. 1). On the other hand, chemicals such as 5-nitroacenaphthene which exhibit approximately 50% of their activity in strains deficient in this esterification enzyme (McCoy et al., 1983) probably form arylhydroxylamine intermediates (or the derived nitrenium ions) which are only half as effective as their corresponding hydroxamic acid esters in reacting with DNA. Obviously, a definitive answer will require the determination of the rates of DNA reactivity of the arylhydroxylamines, the arylnitrenium ions and the corresponding hydroxamic acids esters. Still, the use of specific tester strains may provide an indication of the relative electrophilicities of intermediates in the biotransformation of nitro- and amino-arenes.

A closer look at the bioconversion of nitroarenes in the various Salmonella strains revealed that the esterification enzyme could well be a transacetylase. This activity

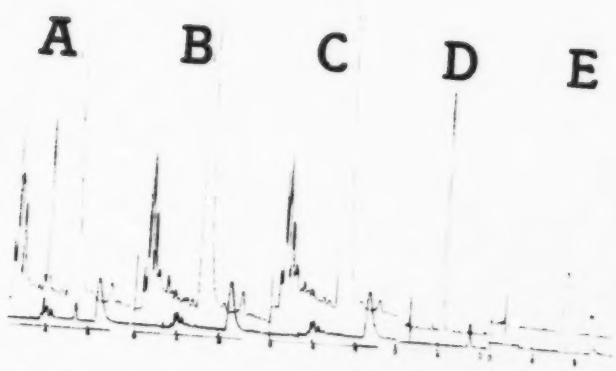

Fig. 3. HPLC profiles of extracts from bacterial cells exposed to 2-aminofluorene: (A) TA100, (B) TA100-Tn5-1,8-DNP1011 and (C) TA100-Tn5-1,8-DNP1012. Panels D and E represent the elution profiles of 2-acetylaminofluorene and 2-aminofluorene, respectively. Note the virtual elimination of the peak representing 2-acetylaminofluorene (retention time 4.0 min, upper curves) in the extracts derived from the 1,8-DNP-resistant strains (B and C). The preparation of the cell extracts and operation of the HPLC column are described in the legend of Fig. 2.

can be readily assayed by using 2-aminofluorene as the acetyl recipient. This led to the development of a simple assay based upon HPLC for recognizing this enzymic activity. Thus while both TA98 and TA98NR form *N*-acetyl-2-aminofluorene from 2-aminofluorene, this product is not made in appreciable quantities in TA98/1,8-DNP$_6$ (Fig. 2).

TA98/1,8-DNP$_6$ was constructed by exposing bacteria to 1,8-DNP and selecting a strain that was resistant to 1,8-DNP (McCoy et al., 1981b), possibly the most potent bacterial mutagen. The potential therefore existed for the introduction of additional mutations and hence the properties of TA98/1,8-DNP$_6$ could be the result of multiple mutations. In order to eliminate this possibility, resistance to 1,8-DNP was introduced by transposon-mediated mutagenesis (Kleckner et al., 1977; Biek and Roth, 1980). This permitted the isolation of 1,8-DNP-resistant strains without the requirement for prior exposure to this chemical. Such strains (isolated as derivatives of TA100) exhibited the same properties of TA98/1,8-DNP$_6$, i.e., blockage in acetylation of 2-aminofluorene (Fig. 3) and a decreased mutagenic response to nitro- as well as nitroso- and *N*-hydroxylamino-arenes (Table 1) but a by-pass of the block by *N*-acetoxy-2-acetylaminofluorene niridazole, NQO and Entozon.

A corollary to these observations is the conclusion that although TA98/1,8-DNP$_6$ and similar esterification-deficient strains are useful in assays designed to detect and identify certain nitroarenes, because of the nature of the enzymic defect such strains will also be non-responsive to the S9-mediated mutagenicity of some aromatic amines, e.g., 2-aminoanthracene, 2-aminofluorene and 2,4-diaminoanisole (the arylhydroxylamine derivatives of which may also require esterification, Table 1). This must be taken into consideration when using these strains to study the behavior of environmental samples in the presence of S9.

This investigation was supported by the National Institute of Environmental Health Sciences and the U.S. Environmental Protection Agency.

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